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Mushroom polysaccharide extracts delay progression of carcinogenesis in mice

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Hepatocellular carcinoma results when cancerous cells are localized in the liver. It is distributed globally with high prevalence in sub-Saharan African, southern Asia, China and Japan. Diagnosis is experimental and in many cases inaccurate due to unreliability of markers. Prognosis is poor and the cost of treatment prohibitive. Conventional radiation and chemotherapy lead to loss of hair, fertility and general weakening of the body's immune system increasing a patient's risk to infection. These observations underscore the need for improved, or additional methods of cancer diagnosis and management. We investigated the effect of polysaccharide rich *Pleurotus pulmonarius* fruit body extracts on progression of chemically induced hepatocellular carcinoma in CBA mice. Addition of *Pleurotus pulmonarius* extracts in diet delayed progression of carcinogenesis suggesting that these extracts may be useful as adjuvants to conventional cancer therapies.

Key words: Carcinogenesis; Mice; Mushroom extracts; Pleurotus pulmunarius

INTRODUCTION

Pleurotus pulmonarius belongs to Basidiomycetes, a class of fungi that produce sexual spores from basidia. They grow on living trees as parasites and on dead wood in forests as saprophytes. They are found in soil as pathogens while some form mycorrhizal association with roots of trees. Some are important pathogens associated with leafy parts of plants as rusts or with flowering structures as smuts. These fungi have medicinal compounds including polysaccharides, terpenoids, sterols and lipids, which are effective in the management of carcinomas (1), leukemia (2), and viral infections (3). Additional reports indicate that soluble polysaccharides from *Lentinula edodes* are effective anti-cancer agents when taken orally (4).

Mushroom polysaccharides are thought to exert anti-tumor effects by activating host immune responses and are according to Wasser and Weis (5), biological response modifiers, capable of helping the body to adapt to various environmental and biological stresses. Although preliminary data suggest efficacy of mushroom extracts as possible adjuvant therapies to cancer, data regarding use of these extracts in clinical management of cancer is not only scanty but anecdotal with conflicting reports on efficacies. There is therefore a need for further investigations into potency of these extracts not only as adjuvant but as primary forms of therapy as well. We investigated the effect of *Pleurotus pulmonarius* polysaccharide rich extracts on progression of chemically induced hepatocarcinogenesis in CBA mice.

MATERIALS AND METHODS

Mushrooms

Mushrooms (*Pleurotus pulmonarius*) were purchased from a farmer in Western Kenya, cut into small slices and dried overnight in a conventional oven at 30°C then crushed to fine powder in a multifunctional grinder.

Extraction of polysaccharides

Polysaccharides were extracted as described by Jamas *et al* (6). Five volumes of 1.0 N NaOH at 85°C was added to 1g of dried mushroom and left to stand for 2 hours then five volumes of 0.5 N acetic acid at 75° C added and agitated for one hour at room temperature. Extracts were centrifuged at 640 *g* for 10 minutes, washed three times with distilled water at 640 *g* then mixed with mouse pellets at a ratio of 1:4 and fed to mice.

Animals used and experimental design

30 CBA mice aged 4 weeks (bred and housed at the animal house, Department of Biochemistry, University of Nairobi) were divided into 3 groups of 10 each comprising 5 males and 5 females. Group 1 mice (negative control) were not exposed to carcinogen and were fed on mouse pellets only. Groups 2 and 3 mice were injected with Diethylnitrosoamine (DENA) and subsequently exposed to DENA in the only drinking water through the 12 weeks of experiment. Group 2 mice (positive control) were fed on mouse pellets only while group 3 mice (experimental) were fed on polysaccharide rich *Pleurotus pulmonarius* extracts mixed with pellets at a ratio of 1: 4. Animal care and handling was carried out in accordance with the acceptable codes of practice at the College of Health Sciences of the University of Nairobi, Kenya.

Induction of hepatocellular carcinoma

Hepatocellular carcinoma was induced using a modification of the method described by Ha et al (7). Each mouse was given a single injection of DENA at a dose of 10µg/kg body weight and were subsequently fed on 0.01% DENA *ad libitum* in the only drinking water from the beginning of week 3 to end of week 12.

Preparation of serum

Mice were bled by tail snipping at the beginning of experiment and at the end of weeks 3, 6, 9 and 12. Blood was diluted to 10% using 0.1Molar phosphate buffered saline, pH 7.0 (PBS), left to stand for one hour at room temperature and kept overnight at 4° C. Serum was decanted and clarified by centrifugation for 30 minutes at 56 *g* and 0.01% (w/v) sodium azide added to 100 µl aliquots and stored at -20° C until use.

Assessment of tumor development and progression

Development and progression of carcinogenesis was assessed by determining activities of tumor markers; lactate dehydrogenase (LDH), sialic acid (SA) and by histological analysis of liver tissue.

Determination of lactate dehydrogenase activities in serum

Lactate dehydrogenase was assayed as described by Wrobleski and La Due (8). To a quartz cuvette, 2.7 ml of a 0.1M phosphate buffer pH 7.4 was added followed with 0.1 ml each of serum and NADH and allowed to stand for 20 minutes at room temperature. 0.1ml pyruvate was added and absorbance read at 340 nm for 10 minutes at 2-minute intervals using Ultrospec II-LKB (Biochrom, Cambridge, England). Activity was calculated using the formula below:

$$\text{Units /ml of serum} = \frac{\text{change in absorbance} \times 1000 \times 0.482}{\text{volume of serum used}}$$

0.482 was derived from molecular extinction of NADH, which is 6.22×10^6 (9), used to convert spectrophotometric units to International Units per litre.

Determination of sialic acid in serum

Levels of sialic acid were measured using a modification of the method described by Shamberger (10). To experimental tubes, 0.1 ml serum, 0.9 ml distilled water and 0.2 ml Erich's reagent were added. Blank tubes contained 0.1 ml serum, 0.9 ml distilled water and 0.2 ml of 1:1 mixture of HCl and H_2O. Tubes were sealed with parafilm, vortexed and incubated at 56°C for 24 hours with occasional agitation and 3 ml of 0.9 M NaCl added, vortexed and centrifuged at 1,400 *g* for 10 minutes. Supernatant was aspirated and optical densities read at 525 nm using Ultrospec II-LKB (Biochrom, Cambridge, England). A standard curve was constructed using commercial free N-acetylneuraminic acid fractions diluted to 10 µg – 40 µg/ml.

Preparation of histological slides

Mice were locally anaesthesized using diethyl ether and sacrificed at the beginning (week 0), and at the end of weeks 6 and 12. Liver was cut into small

pieces (1 cubic centimetres) and fixed in bouin's reagent (75 ml -Picric acid, 25 ml Formalin and 5 ml Acetic acid) for 48 hours at room temperature. Tissues were transferred and kept for 90 minutes each in 70%, 80%, 90%, and absolute ethanol (two changes), and in xylene (two changes). Tissues were impregnated with pure molten paraffin wax (50-60°C), then mounted on labeled paper boats and left to solidify at room temperature for 24 hours floated on cold water to hasten solidification then sectioned to 7 microns using a microtome.

Sections were put on microscope slides and dried in an incubator at 37°C for 24 hours; stained with hematoxylin-Eosin stains then hydrated by immersing for 5 minutes in two changes of xylene, absolute, 90%, 80% and 70% ethanol respectively and finally hydrated with water, then stained with Haematoxylin (20-30 min). Excess dye was washed with tap water and tissue differentiated by a quick dip in HCl-alcohol (200 ml 70 % ethanol; 2 ml 36 M HCl) and counterstained in Eosin stain for 3 minutes at room temperature. Tissues were dehydrated by immersing for 5 minutes in 50%, 70%, 80%, 90%, and in two changes of absolute ethanol, xylene 1 and finally xylene 2. A thin layer of DPX mountant was applied and tissues covered with a cover slip.

Histological Examination

Tissues were examined under a microscope (Ortholux, Leitz-Wetzler, Germany) and photographs taken at X 125 magnification.

RESULTS

In the current study, we induced hepatocellular carcinoma in 4-week old CBA mice using a chemical carcinogen, Diethylnitrosoamine (DENA) and monitored progression of carcinogenesis by measuring levels of LDH, sialic acid, and by histological analysis of liver tissue. Potency of mushroom extracts as anti-tumor agents was determined by feeding carcinogen exposed mice on pellets mixed with polysaccharide rich *Pleurotus pulmonarius* extracts.

Administration of carcinogen led to a consistent increase in mean LDH levels, through the 12 weeks of experiment in carcinogen-exposed mice fed on pellets alone. These levels decreased through the first six weeks and increased thereafter to week 12 in mice that were fed on pellets and *Pleurotus pulmonarius* extracts, (Figure 1). Mean sialic acid levels increased through the first nine weeks and decreased thereafter to

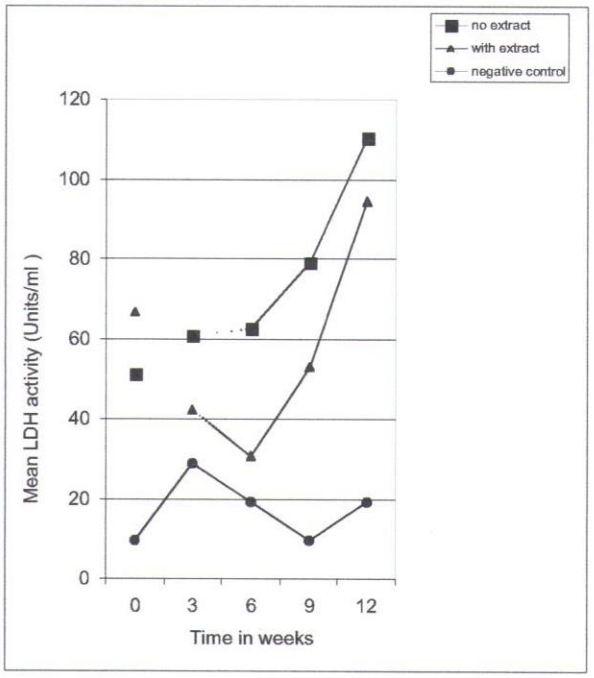

Figure 1. Trends of mean LDH activities. Samples were taken at the beginning of experiment and at end of weeks 3,6,9 and 12.

week 12 in carcinogen-exposed mice fed on pellets alone but decreased consistently through the 12 weeks in those fed on pellets and *Pleurotus pulmonarius* extracts (Figure 2). Both LDH and sialic acid levels were significantly higher in experimental mice compared to negative controls (Figures 1 and 2).

Using histological analysis, we demonstrated that carcinogenesis had fully developed at the end of week 12, and that liver tissue from carcinogen exposed mice fed on pellets alone (Figure 3b) had pale bodies and large nuclei with relatively lower degree of chemosensibility. On the contrary, tissue from carcinogen-exposed mice fed on pellets and *Pleurotus pulmonarius* extracts (Figure 3c) had prominent nucleoli with marginalized chromosomes closely resembling those of normal healthy tissue. Overall, these results show that polysaccharide-rich *Pleurotus pulmonarius* extracts delayed progression of carcinogenesis, that sialic acid is reliable as a marker of cancer related tissue injury and that LDH works in a smaller window of time.

DISCUSSION

Polysaccharides are among the best known mushroom extracts with potent anti-tumor and immunomodulating properties (11, 12). In the current study, we have used histological analysis to demonstrate that inclusion of polysaccharide rich *Pleurotus pulmonarius* extracts in diet delays progression of hepato-carcinogenesis in CBA mice. The observation that levels of sialic acid and not those of LDH decreased consistently through the 12 weeks in mice fed on pellets and *Pleurotus pulmonarius* extracts, suggests that sialic acid is more reliable as a marker of cancer related tissue injury compared to LDH.

Our observations agree to some extent with earlier reports of Zhuang et al (13) that *Pleurotus pulmonarius* extracts have anti-tumour or immune modulating properties. These results must however be viewed in the context of other reports (14) that mushroom extracts have no clear inhibitory activity on hepatoma. That such disparities may result from minor differences in experimental set up, purification procedures and heterogeneity of animal models used cannot be ruled out. However, these discrepancies also highlight the need for well-controlled laboratory experiments in addition to randomized double-blinded clinical trials with appropriate placebo treatments.

Additional reports indicate that Krestin (PSK), a preparation consisting predominantly of glucans and approximately 25% protein from *Coliolus versicolor* restores immune responses of tumor bearing animals,

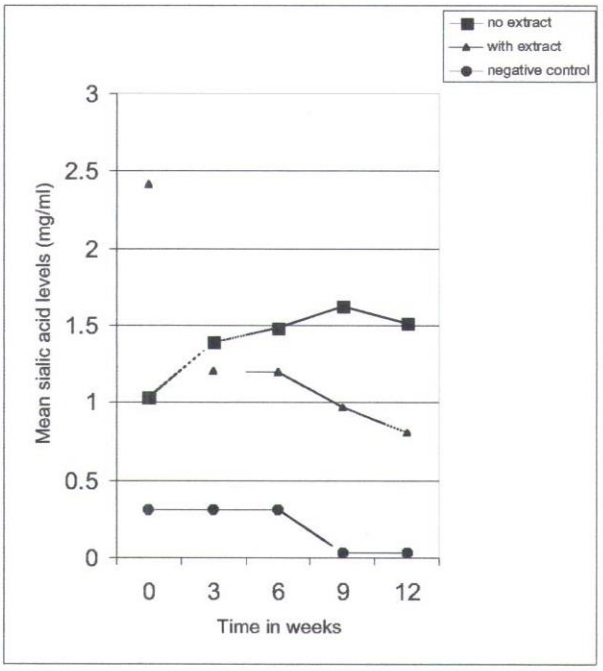

Figure 2. Trends of mean sialic acid activities. Samples were taken at the beginning of experiment and at end of weeks 3,6,9 and 12.

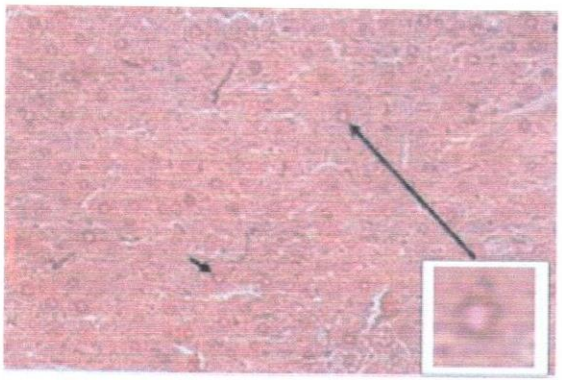

Figure 3a. Histological appearance of healthy mouse liver tissue. Mice were not exposed to carcinogen and were fed on pellets only (negative control). They were sacrificed at the end of week 12 and liver samples processed for histological analysis. Tissue had prominent nucleoli and marginalized chromosomes (see insert) characteristic of healthy liver tissue (original magnification: x125).

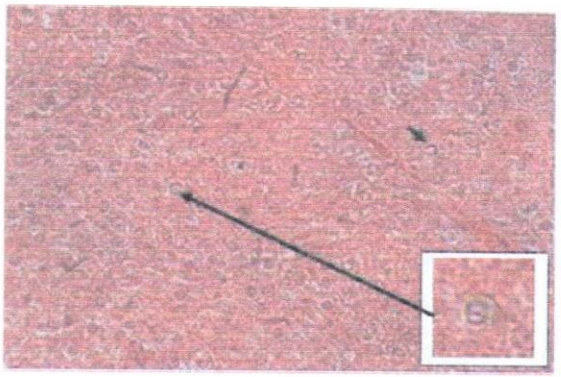

Figure 3c. Histological appearance of moderately differentiated mouse liver tissue. Mice were exposed to carcinogen and fed on pellets and Pleurotus pulmonarius extracts. They were sacrificed at the end of week 12. Tissue had prominent nucleoli and marginalized chromosomes (see insert) closely resembling those of healthy liver tissue (original magnification: x125).

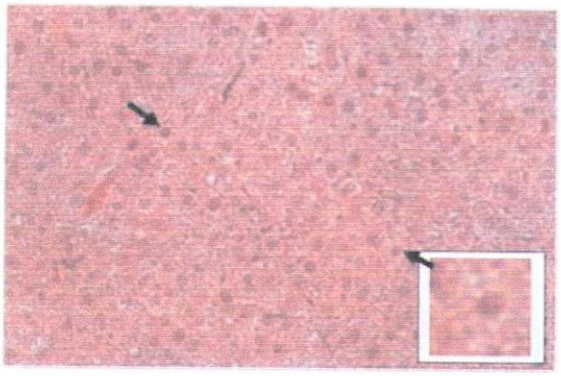

Figure 3b. Histological appearance of well differentiated mouse liver tissue. Mice were exposed to carcinogen and fed on pellets only. They were sacrificed at the end of week 12 and liver samples processed for histological analysis. Tissue had pale bodies and large nuclei (see insert) with relatively lower degree of chemosensibility in the cells characteristic of carcinogenesis (original magnification: x125).

and is effective as an adjuvant therapy (15). When used in addition to radiation therapy for stage III non-small cell lung cancer, the 5-year survival was 22% compared to only 5% in controls (16). On the contrary, Suto et al (17) reported no survival benefits to PSK in hepatocellular carcinoma patients after treatment with various standard therapies. Elsewhere, Haranaka et al (18) reported that krestin did not show anti-tumor activities when tested for TNF production. This assertion can however be challenged on the basis that anti-cancer agents may in addition to TNF production exert their effects via yet to be determined modes.

The desperation among cancer patients is highlighted by a recent report on Nature news 03/28/2007 (19) titled "cancer patients opt for unapproved drug" citing use of dichloroacetate (DCA) by patients in United States. That such practices can undermine performance of controlled clinical trials, and expose patients to undesirable side effects cannot be overstated. Undeniably however, these patients have no luxury of waiting for the often time consuming clinical trials and certification of drugs for use. Consequently, there is a need to develop and rapidly avail to patients the newly acquired therapies.

CONCLUSION

Despite the recent advances in research into medical sciences, cancer related morbidity and mortalities continue globally. That mushroom extracts have shown promising results is exciting. However, data relating to their use in clinical management of cancer is not only scanty but anecdotal with conflicting reports on efficacies. In the light of our observations and previous reports reviewed herein and elsewhere, we suggest that polysaccharide rich *Pleurotus pulmonarius* extracts be used in combination with other forms of therapy to cancer in well-controlled clinical trials with appropriate placebo treatments. In these studies, caution should be exercised to avoid potential toxicities

arising from mistaken identities. In addition, efforts should be made by all concerned to ensure faster availability of newly acquired therapies to patients.

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